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# Binding of Adenovirus to Its Receptors in Mouse Astrocytes Induces *c-fos* Proto-Oncogene and Apoptosis

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We have demonstrated that Ad.βGal, a broadly used adenoviral vector of serotype 5, binds and induces proto-oncogene *c-fos* expression in quiescent cultures of mouse brain astrocytes. As observed in Northern blots, the expression of this immediate early gene is induced by viral infection in a dose-dependent manner, peaking at a multiplicity of infection (m.o.i.) of 100. The expression of *c-fos* is transient, being maximal after 30 min and disappearing 2 h after infection. A previously reported method was used to study the presence of receptors for adenovirus in the cellular membrane of murine astrocytes. After absorption of the virus, rabbit antibodies and $^{125}$I-protein A were used to form a sandwich on the cellular surface, and 9000 adenovirus-specific receptors were demonstrated on each astrocytic cell. Binding was temperature dependent and reached a plateau after 60 min. The specificity of *c-fos* induction is demonstrated by its neutralization by anti-adenovirus-specific antibodies. Although clear apoptosis cannot be demonstrated *in vitro* by DNA laddering, maybe due to a lack of sensitivity of the method, a statistically significant increase in caspase-3 activity is demonstrated in astrocyte cultures infected at a m.o.i. of 100 by adenovirus. Furthermore, a perfect colocalization is shown *in vivo* between cells infected with the Ad.βGal vector and apoptotic astrocytes, as demonstrated by TdT-mediated dUTP nick end labeling (TUNEL) staining. The purpose of our study was to ascertain the potential for adenovirus as a gene therapy vector for neural disorders caused by astrocyte dysfunctions. 

## INTRODUCTION

Wild-type adenoviruses naturally infect epithelial and other resting cells. The actual clinical usefulness of gene therapy depends on the development of recombinant viral vectors mainly belonging to the human adenovirus serotype 5 (Ad5). This nonpathogenic virus, which contains a 36-kb double-stranded DNA genome, can be grown and purified in suitable quantities (Neve, 1993). It has a broad tropism, which is essential for its use in gene therapy, and can infect nondividing cells, such as central nervous system (CNS) cells (Berkner, 1988). A lack of knowledge on the precise mechanisms by which adenovirus binds susceptible CNS cells remains, although it has been recently reported that group D adenoviruses infect primary CNS culture cells more efficiently than those from group C (serotypes 2 and 5) (Chillon *et al.,* 1999).

The capsid of adenovirus has a projection, the fiber, containing three structural domains. One of these domains, called knob, which contains the C-terminus of the protein, interacts with the cell surface receptors. Therefore, the fiber knob is considered the domain responsible for the primary event of binding the adenovirus to its cell membrane receptor (Choboczek *et al.,* 1995; Weber *et al.,* 1989). In the cellular counterpart, recent studies have demonstrated that CAR, a 46-kDa host cell membrane protein, serves as the primary receptor for serotypes 2 and 5 adenoviruses as well as for Coxsackie B virus (Bergelson *et al.,* 1997).

The broadly used vector Ad.βGal infects primary cultures of rat astrocytes, as well as other CNS cell populations, as determined by blue nuclear staining (Le Gal La Salle *et al.,* 1993; Ridoux *et al.,* 1994). We undertook this study to characterize the binding of the adenoviral vector Ad.βGal to mouse astrocytes, and to determine if the binding of the viral particles to its receptor/s induces the expression of the early response gene *c-fos* and/or apoptosis.

A basal expression of *c-fos* has been demonstrated in the cerebral cortex of rodents (González-Martín *et al.,* 1991, 1992). Adenovirus and the adenoviral E1A protein have been reported to activate the expression of the Fos-Jun AP-1 dimers in F9 mouse teratocarcinoma cells (Hagmeyer *et al.,* 1993) in a similar way to Visna virus (Shih *et al.,* 1992), the proteins of hepatitis virus (Twu *et al.,* 1993), or Theiler's murine encephalomyelitis virus (TMEV) (Rubio *et al.,* 1996; Rubio and Martin-Clemente, 1999) in other cellular systems.

Continuous *c-fos* expression precedes programmed cell death or apoptosis in the CNS (Smeyne *et al.,* 1993). Recently, the induction of apoptosis in turkey spleen cells by pathogenic avian adenovirus type II has been

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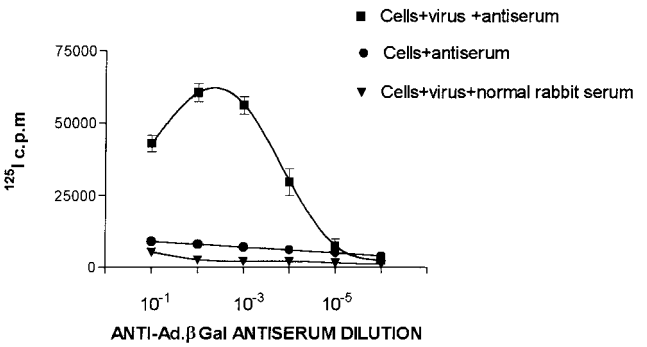


FIG. 1. Titration of rabbit antiserum against cesium chloride-purified Ad.βGal viral particles. Tenfold dilutions of antiserum or preimmune normal rabbit serum were tested in the immunoassay on confluent cultures of astrocytes uninfected or infected at a m.o.i. of 100.

reported (Rautenschlein *et al.,* 2000) and the "altruistic suicide" of immature neurons infected by the alphaviruses Semliki Forest virus and Sindbis virus is a well-know phenomenon (Allsopp and Fazakerley, 2000). Therefore, we investigated if the induction of *c-fos* or the Ad.βGal infection triggers programmed astrocyte death per se, both *in vitro* and *in vivo* by using DNA laddering, caspase-3 activity, and TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated nick end labeling) staining. The demonstration of *in vivo* apoptosis induction, as is the case, could have important implications in the future success of adenovirus-mediated gene therapy.

## RESULTS

### Antiviral antiserum titration

A rabbit was immunized with highly purified virion particles to generate a polyclonal antiserum containing antibodies against adenovirus serotype 5 capsid proteins. The titer of such antiserum (dilution producing 50% of the total $^{125}$I-labeled protein A binding) is 1:10,000 (Fig. 1). Therefore, the antiserum was used at a 1:1000 dilution throughout this work. Normal preimmune rabbit serum tested at any dilution, or omission of viral particles, produced only background binding of the label (Fig. 1). These results show that our immunoassay is highly specific and that the attachment of adenoviral particles to the cell receptor/s does not inhibit the subsequent binding of antibodies to the virions.

### Binding of Ad.βGal to astrocytes

The multiplicity of infection needed to achieve saturation of the surface receptors of formaldehyde-fixed cells is shown in Fig. 2. Tenfold dilutions of the virus stocks were allowed to bind to astrocyte monolayers ($1 \times 10^6$ cells per 35-mm-diameter petri dish) for 30 min at 37°C. The excess virus was removed by washing the monolayers twice. After fixation with 3% formaldehyde in PBS, cultures were exposed to antiserum at 1:1000 dilution and to $^{125}$I-labeled protein A. Bound radioactivity was estimated after solubilization with 2% SDS. The amount of labeled protein A attached to the cells was proportional to the number of viral particles added to the monolayers and saturation was achieved at 500 plaque forming units (PFU) per cell. Omission of virus produces background levels of radioactivity due to the nonspecific adherence of the label to the petri dishes. On the other hand, fixation with formaldehyde preserves to a great extent the functionality of the cell surface receptors. The saturation of the binding capacity of astrocytes takes place at a m.o.i. of 500 PFU per single cell. We have measured by electron microscope counts and plaque assay that in our Ad 5 stock it takes 18 virion particles (binding a single receptor each) to produce one PFU. This number is very similar to 20 virion:PFU reported for adenovirus type 5 by Green *et al.* (1967). Then, we concluded that there are around 9000 functional receptors per astrocyte.

The specificity of virus binding was demonstrated by the fact that Vaccinia virus infection at a m.o.i. of 500 downward, used as unrelated negative control, does not bind any radioactivity (Fig. 2).

### Kinetics of binding

The time course of the attachment of adenovirus at a m.o.i. of 100 to living, nonfixed astrocytic cells was measured at different temperatures (Fig. 3). Maximum binding was reached very fast, taking place at 30 min at both 37 and 4°C. Interestingly, this is the same period of time required for maximal *c-fos* induction (Fig. 5). The same amount of viral particles seem to be available at the surface of the cells with increasing time, probably because additional virions replace the internalized particles at the astrocyte surface. It is known that in the adenovirus system, attachment of the virus to its cell

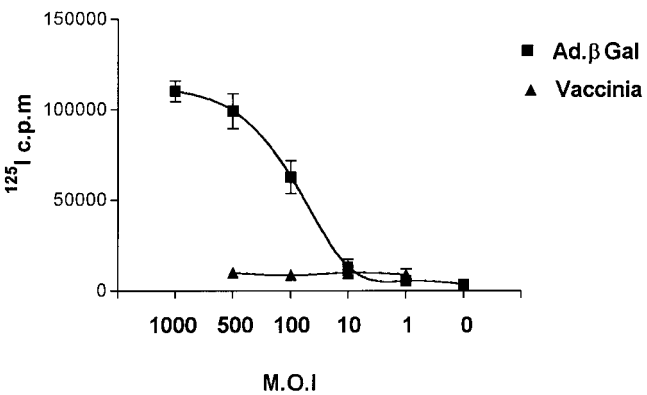


FIG. 2. Saturation of the binding of purified Ad.βGal viral particles by astrocytic cells. Different dilutions of the virus stocks were absorbed for 30 min at 37°C for astrocytes and treated with the rabbit anti-Ad.βGal antiserum diluted 1:1000 and with $^{125}$I-labeled protein A, as described under Materials and Methods. Negative virus control was provided by the binding of Vaccinia virus at m.o.i. of 500 downward.

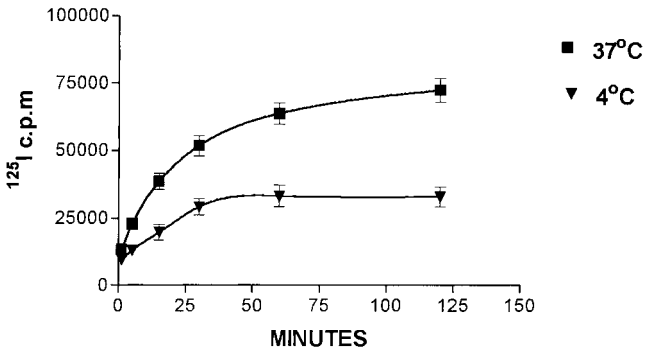


FIG. 3. Kinetics of the binding of Ad.βGal particles at a m.o.i. of 100 to pure astrocytic cultures at 37 and 4°C. The availability of viral particles on cultured cells was measured at both temperatures for different periods of time. The $^{125}$I-protein A bound to the cells at times ranging from 1 to 120 min was estimated after solubilization with 2% SDS.

surface receptor occurs at 4°C, whereas endocytosis requires 37°C (Hong *et al.,* 1997). Here we show that binding to the receptor is much more efficient at 37 than at 4°C (Fig. 3).

### Adenoviral transfection of astrocytes

In order to evaluate the ability of our Ad.βGal vector to transfer and express genes in our pure mouse astrocyte cultures, the cells were stained for β-galactosidase 24 h after infection, as stated under Materials and Methods. Expression of β-galactosidase was readily detected in the majority (74%) of astrocytes infected with Ad.βgal at a m.o.i. of 100, demonstrating that successful gene transfer and expression can be achieved using our vector. Fourteen percent of cells expressing β-galactosidase were found when infected at a m.o.i. of 10 and 78% at a m.o.i. of 500 (not shown). On the basis of these preliminary studies, all subsequent infections were carried out at a m.o.i. of 100. Mock-infected cells showed only background levels of β-galactosidase staining.

### Induction of *c-fos* mRNA in astrocytes by adenovirus

To examine the effect of adenovirus infection on *c-fos* proto-oncogene expression, astrocytes kept in culture for 10–12 days were infected at different m.o.i. and times and total RNA were purified and analyzed by Northern blot. *c-fos* mRNA was not detected in confluent astrocyte cultures rendered quiescent by FCS starvation (Rubio, 1997). However, when infected with Ad.βGal, *c-fos* mRNA levels increased in a dose-responsive manner, peaking at a m.o.i. of 100. Transcripts above background could be detected down to a m.o.i. of 1 (Fig. 4). The system seems to be saturated at a m.o.i. of 100 as a further 10-fold increase did not augment the levels of transcription (not shown). When the relative amount of mRNA was quantified with a Model 300 A computing densitometer (Molecular Dynamics Inc., Sevenoaks, Kent, UK), it was

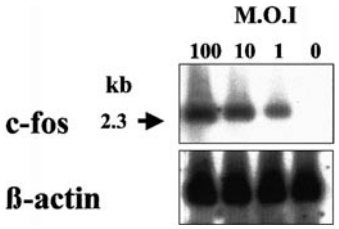


FIG. 4. *c-fos* mRNA levels in mock-infected (0) or astrocytes infected at a m.o.i. of 100, 10, or 1, for 30 min at 37°C as detected by Northern blot. The size of the transcript is indicated on the left. Hybridization to the housekeeping gene β-actin was used to verify equal RNA loading.

shown that the bands obtained after infection at m.o.i. of 100, 10, and 1 are 8, 6, and 4 times, respectively, more dense than the background. Hybridization to a human housekeeping β-actin probe (Clontech) served as an internal control for loading throughout the experiments (Figs. 4 and 5).

### Time-course induction of *c-fos* mRNA

The time required for astrocytes to induce *c-fos* was examined by infecting cells at an optimal m.o.i. of 100 for periods of time ranging from 30 to 120 min. The 2.3-kb *c-fos* mRNA transcript was transiently and rapidly induced, showing maximal induction after 30 min. Its expression decreased after 1 h with no *c-fos* mRNA being observed 2 h after infection (Fig. 5), as is the case at 0 min time (not shown). The bands obtained 30 and 60 min after infection were respectively 10 and 8 times more dense than that obtained at 120 min.

### Adenovirus infection is the inducer of oncogene expression

We next ruled out that growth factors present in the culture medium along with the Ad.βGal samples were responsible for the induction of proto-oncogene expression. In order to do so, we determined the capacity of different dilutions of our specific anti-adenovirus antiserum (dilutions ranging from $10^{-2}$ to $10^{-5}$) to neutralize the above induction. As shown in Fig. 6, incubation with

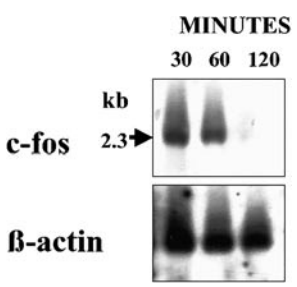


FIG. 5. Time course of *c-fos* mRNA induction in astrocytes by Ad.βGal at a m.o.i. of 100. Cells were infected for periods of 30, 60, and 120 min. Total RNA was further purified and analyzed by Northern blot. Hybridization to β-actin was used to verify equal loading.

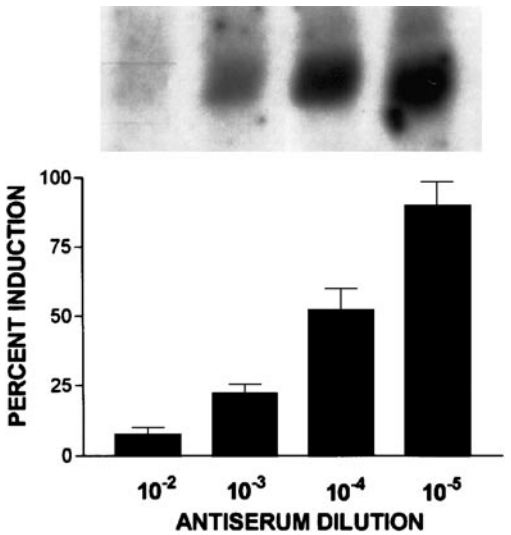


FIG. 6. Inhibition of the Ad.βGal-induced expression of c-fos mRNA by specific anti-adenovirus antibodies. Purified virus samples were preincubated for 30 min at 37°C with different dilutions ($10^{-2}$ to $10^{-5}$) of rabbit antiserum and then added to the astrocytic cultures. After 30 min, mRNA was extracted and analyzed by Northern blot. Induction obtained without antiserum was considered as 100% in densitometric analysis. The autoradiogram represents a typical response from a set of triplicate samples.

specific antibodies completely abated the induction of *c-fos* mRNA, demonstrating that proto-oncogene expression is dependent upon binding of adenovirus by astrocytes. In contrast, when preimmune normal rabbit serum was incubated with the viral particles at the same dilutions, the inducing capacity of adenovirus did not change (not shown).

## Apoptosis induction in culture

Overexpression of some genes involved in cell growth signaling as *c-jun* (Bossy-Wetzel *et al.,* 1997) or *c-fos* (Hu *et al.,* 1996) has been reported to cause apoptosis. Therefore, we examined if adenovirus infection, as an inducer of *c-fos* overexpression, is also an inducer of apoptosis in astrocytic cells. The infection of astrocytes at a m.o.i. of 100, 10, and 1 did not induce clear DNA laddering when tested 24 h later. Positive laddering controls were provided by U937 cells treated with camptothecin, included in the apoptotic DNA-ladder kit used, and FCS-deprived astrocytic cells (not shown). Because the agarose gel technique may lack sufficient sensitivity, measurement of changes in caspase-3 levels was used as a second method to detect programmed cell death. Caspases participate in a cascade of proteolytic cleavage events in dying cells and caspase-3 (CPP32), is a member of such a caspases family with a substrate specificity for the amino acid sequence (Asp-Glu-Val-Asp) (Nicholson *et al.,* 1995).

Figure 7 demonstrates that FCS starvation induces apoptosis in astrocytes (Fig. 7, +) as compared with a negative control provided by mock-infected cell cultures (Fig. 7, −). Astrocyte cultures infected with a m.o.i. of 100 for 24 h are the only ones showing a statistically significant increase in caspase-3 activity. This induction was specific because the lysates from cells treated with the irreversible, pan-caspase inhibitor Z-VAD-FMK at a final concentration of 50 μM, added directly to the astrocytic cultures infected at a m.o.i. of 100, do not show appreciable caspase-3 activity over the negative mock-infected astrocytes. The specific activities are a mean of 41.6 pmol/μg for astrocytes infected at a m.o.i. of 100 and 24.3 pmol/μg for untreated cells. FCS-starved cells produce a mean of 95.3 pmol/μg of protein in cell lysates.

## *In vivo* induction of apoptosis

Two regions, anterior hypothalamus and septum, were mainly studied to evaluate the ability of the adenoviral vector to infect astrocytes *in vivo* 4 days after intracerebral injection. Labeled cells were found as far as 1.5 mm from the needle track. Profuse red nuclei staining was detected (Fig. 8B) by the sensitive method of using an antibody against the enzyme instead of X-gal substrate staining (Le Gal La Salle *et al.,* 1993). To investigate if these cells were undergoing apoptotic death, TUNEL staining was performed on serial sections (Fig. 8A, green cells) showing a perfect colocalization with β-galactosidase-positive cells (Fig. 8C, yellow cells). When a more quantitative analysis of the costained cells was performed using the Analytical Imaging Station (Imaging Research Inc., Canada) a mean of 11% of the cells was costained for both TUNEL and β-galactosidase. A small amount of residual necrosis was found surrounding the

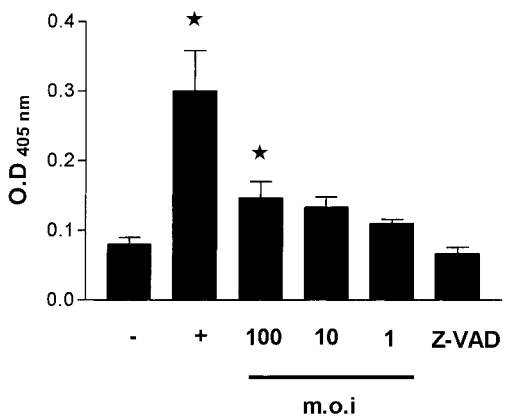


FIG. 7. Measurement of caspase-3 activity in Ad.βGal-infected astrocyte cultures. Astrocytes were mock-infected (−), deprived of FCS for 1 week as positive apoptotic control (+), or infected at a m.o.i. of 100, 10, or 1. Cells infected at a m.o.i. of 100 were treated at the same time with the irreversible, pan-caspase inhibitor Z-VAD-FMK at a final concentration of 50 μM (Z-VAD). Cell extracts were tested for caspase-3 activity according to the assay conditions of the CaspACE kit. Every enzymatic reaction contained 50 μg of total protein cell extract. Error bars indicated SD of three different experiments. Statistically significant values (*) were calculated by Student's *t* test.

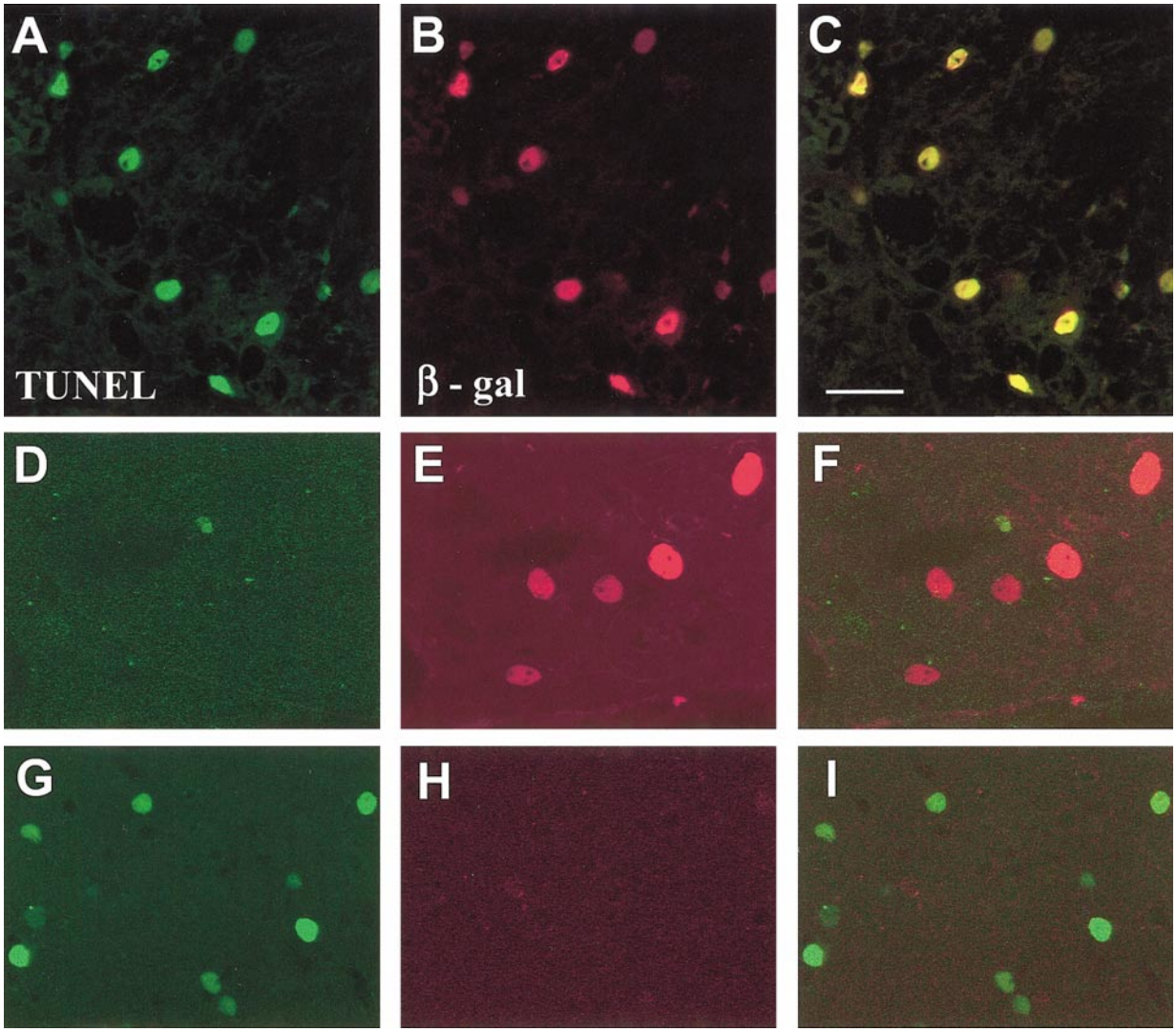


FIG. 8. Double staining confocal images of astrocytes stained for TUNEL (A, green nuclei) $\beta$-galactosidase (B, red nuclei) at the septal region level, 4 days after Ad.$\beta$Gal virus injection. Both labelings colocalize exactly at the composite image in the same astrocytes shown whose nuclei, but not the nucleoli, were positive (C, yellow nuclei). D–F images show another infected mouse brain section containing a negative control for TUNEL (without terminal deoxynucleotidyl transferase added (D), stained for $\beta$-gal (E), and a composite image (F). G–I images show a third representative section containing TUNEL staining (G), a negative control for $\beta$-gal, where primary antibody was omitted (H), and a composite image (I). Scale bar is 30 $\mu$m for A–C and G–I and 20 $\mu$m for D–F.

point of injection in brains of control 6-week-old mice injected with 5 $\mu$l of PBS, but no TUNEL staining was detected. Therefore, the apoptosis observed *in vivo* after viral inoculation is attributed to adenoviral infection of the target cells, which are astrocytes as determined by glial fibrillar acidic protein (GFAP) staining (not shown). Negative controls, including sections where TdT for TUNEL (Fig. 8D) and anti-$\beta$-gal antibody (Fig. 8H) were omitted, were clearly negative. Although most apoptotic cells are astrocytes according to such staining (around 80%), other cells with morphological and anatomical characteristics of neurons and microglial cells were stained for $\beta$-galactosidase, but not for TUNEL (not shown).

## DISCUSSION

The goal of the present study was to identify and quantify the receptors for the adenovirus 5 Ad.$\beta$Gal on the cell surface of cultured mouse astrocytes and to determine if the binding induces some biological response, such as proto-oncogene *c-fos* expression and/or apoptosis. Preliminary studies showed that Ad.$\beta$Gal infects nondividing neural cells, both neurons and astrocytes, *in vivo* and *in vitro* (Le Gal La Salle *et al.,* 1993; Ridoux *et al.,* 1994). Several cellular genes, such as proto-oncogenes, can be activated during infectious virus gene expression. Here we report the quantification of receptors for adenovirus serotype 5 on mouse astrocytes and the induction of *c-fos* and apoptosis that rapidly and

specifically follows the binding on viral particles to its 9000 membrane receptor/s per cell.

The possession of cell surface receptors determines the host range for viruses. We have used in this study a method previously described by us to study the characteristics of binding of purified Theiler's murine encephalomyelitis virus to cells, using antibodies as specific probes and then detecting binding with $^{125}$I-labeled protein A (Rubio and Cuesta, 1988). The binding was specific, as demonstrated by the fact that no binding of the $^{125}$I-labeled staphylococcal protein A was detected when virus, antibodies, or cells were omitted. This assay allows us to study the early interactions of an adenoviral vector to mouse astrocytes via its specific receptor/s. Nevertheless, we cannot distinguish between two different receptors, as HeLa cells use to bind serotype 5 adenovirus (Stevenson *et al.,* 1995). The saturation of the binding capacity reached a maximum plateau of 500 PFU per cell (Fig. 2). As in our adenovirus stock it takes 18 virion particles to generate one PFU, we calculate the number of 9000 receptors per astrocytic cell. This figure is similar to those previously calculated by other methods for a range of host cells, where the number of receptors available for Ad 5 binding varies from 5 to 10 $\times$ $10^3$ per cell (Doerfler and Bohm, 1995; Persson *et al.,* 1985; Defer *et al.,* 1990).

We also found that adenovirus attachment to its receptor is temperature dependent. It took place at 37°C but reached low levels at 4°C (Fig. 3). Adenovirus serotype 5 (hAd5) entry into host cells depends on integrins, a family of proteins that mediate cell adhesion and motility of the extracellular matrix, mainly $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins (Huang *et al.,* 1995; Wickham *et al.,* 1993), and is regulated by the rab5 GTPase (Rauma *et al.,* 1999). We have not determined the level or composition of integrins of our mouse astrocyte cultures but Previtali *et al.* (1997) reported the absence of $\alpha$V integrin, as well as $\alpha 2$–$\alpha 5$, $\beta 3$, and $\beta 4$, in rat cultured astrocytes. The only integrins found by immunocytochemistry by these authors were $\alpha 1$, $\alpha 6$, and $\beta 1$. Higher expression of $\alpha$V in rat astrocytes *in vivo* was detected during the course of experimental autoimmune encephalomyelitis (EAE) (Previtali *et al.,* 1997).

Expression of *c-fos* cannot usually be detected in the nuclei of normal glial cells, although heat stress and brain lesions induce massive Fos immunoreactivity in glia *in vivo* (Dragunow and Robertson, 1988; Dragunow *et al.,* 1989). Overexpression of both *c-fos* and *c-jun* has been reported by our group to be induced in the glioma cell line C-6 as well as in astrocytes after TMEV infection (Rubio *et al.,* 1996; Rubio and Martin-Clemente, 1999) or after IFN-$\gamma$ treatment (Rubio, 1997). The infection of cultures of mouse cortical astrocytes with the adenoviral vector Ad.$\beta$Gal resulted in *c-fos* mRNA accumulation that reached a maximum at 30 min, the time needed to reach maximum binding of virions (Figs. 3 and 5). A rapid decline followed. This rapid kinetics of proto-oncogene induction shows that the stimulatory effect was induced early upon binding of adenovirus to its cellular receptor. The expression of mRNA depends on the m.o.i. tested, the optimal being 100 (Fig. 4). Specific antiviral antibodies, the same used in the binding test, suppress *c-fos* induction when allowed to cover the viral particle prior to receptor binding (Fig. 6). This demonstrates that the effect found is due to viral attachment and not to any growth factor present in the culture medium.

Apoptosis induction is also detected in astrocytic cells infected at a m.o.i. of 100 by our adenoviral vector when a technique as sensitive as the detection of caspase-3 activity is used (Fig. 7). Despite the fact that no significant change in the infected brains is indicated by histological examination, the *in vivo* situation shows a clear apoptosis, as detected by TUNEL staining, in all nuclei of astrocytic cells infected by the Ad.$\beta$Gal vector (Fig. 8). This positive population undergoing apoptosis corresponds to a significant number of cells in each field examined.

There is an increasing interest in gene therapy, based on corrective genetic material delivery into "sick" cells by using different viral vectors as adenoviruses (Verma and Somia, 1997). Recent success in prevention of experimental autoimmune encephalomyelitis, an experimental model for multiple sclerosis, has been reported by the expression of Il-10 (interleukin-10) drived by an adenovirus vector (Cua *et al.,* 2001). Nevertheless, the injection of adenoviral vectors causes inflammation in the brain (Byrnes *et al.,* 1995) and this rapid inflammatory response is mediated by Il-1 (interleukin-1) (Cartmell *et al.,* 1999). The biological relevance of our findings is the demonstration of the induction of apoptosis in a population of brain cells, the astrocytes, infected by a first generation (E1/E3 deleted) adenoviral vector. Despite a collateral possible benefit from the described reaction in the treatment of astrocytomas, this is a problem that remains to be overcome further before gene therapy becomes a routine practice in the treatment of the brain-associated diseases.

## MATERIALS AND METHODS

### Astrocyte cultures

Astrocyte cultures were prepared by mechanical dissociation of the cerebral cortex from newborn Balb/c mice (Rubio *et al.,* 1990). The cortex was isolated under a dissecting microscope and cleaned of choroid plexus and meninges. Cell suspensions were filtered through and 80-$\mu$m-pore-size mesh into Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS) and gentamycin (Flow Laboratories, Irvine, UK). After centrifugation, cells were filtered through a 20-$\mu$m mesh and cultured at 37°C. The medium was changed after 4 days in culture and subsequently two times a week for the entire culture period. Cultures were en-

riched in astrocytes by the removal of less adherent oligodendrocytes by shaking for 18 h at 37°C and 250 rpm in a G24 environmental shaker (New Brunswick Scientific, Edison, NJ). Cellular confluence was observed 10 days after plating, with cells showing a polygonal flat morphology. A mean of 98% astrocytes was confirmed by indirect immunofluorescence staining of methanol-fixed cultures using rabbit anti-glial fibrillar acidic protein antiserum (Dakopatts, Glostrup, Denmark). The lack of noticeable mature oligodendrocytes and microglial/macrophage cells was determined using a guinea pig anti-myelin basic protein (MBP) antiserum prepared as described elsewhere (Rubio *et al.,* 1990) and monoclonal anti-Mac-1 antibody (Serotec, Oxford, UK). Secondary fluorescein-labeled antibodies were purchased from Sigma Chemical Co. (St. Louis, MO).

### Adenovirus

The adenovirus Ad.$\beta$Gal, belonging to the serotype 5 and harboring the *LacZ* gene of *Escherichia coli* coding for $\beta$-galactosidase under control of the Rous sarcoma virus promoter, was used throughout this work. It has been deleted of sequences E1 and E3, impairing the ability of this viral construct to replicate (Stratford-Perricaudet *et al.,* 1992). The virion particles were purified by CsCl banding, desalted on PD-10 desalting columns (Pharmacia Biotech, Sweden) eluted in PBS, aliquoted, and stored at −70°C in PBS-10% glycerol. The titers of the viral stocks were determined by plaque assay using the human embryonal kidney cell line 293. Levels of endotoxin contamination were below 0.2 U/ml in all virus preparations used.

A control Vaccinia virus stock ($2 \times 10^8$ PFU/ml) was provided by Dr. Eduardo Paez, Centro de Investigaciones Biologicas, Madrid, Spain.

### $\beta$-Galactosidase detection

The expression of the *LacZ* gene after the infection of the astrocyte cultures in 35-mm-diameter petri dishes by the Ad.$\beta$Gal viral vector was assessed by staining with an anti-$\beta$-galactosidase antibody (Sigma) as described under Immunocytochemical Methods.

### Antiserum production

Antiserum was produced in New Zealand White rabbits by subcutaneous and intramuscular injections at multiple sites. Four injections containing 100 $\mu$g each of CsCl-purified Ad.$\beta$Gal virus inactivated for 60 min at 56°C and emulsified with complete Freund adjuvant (Difco Laboratories, Detroit, MI) were given at 2-week intervals. The rabbits were bled 12 days after the last booster, and serum was frozen at −20°C until used.

### Immunoassay for virus binding

Monolayers of astrocytes in 35-mm-petri dishes (Costar, Cambridge, MA) were infected with 100 $\mu$l of the virus dilutions in DMEM containing 0.1% BSA (Sigma) for 30 min at 37°C as previously reported (Rubio and Cuesta, 1988). After three washes, virus-coated cells were fixed for 30 min at room temperature in freshly prepared 3% formaldehyde in PBS. After washing, the cells were incubated with diluted antiserum at 37°C for 30 min, and after two more washes, the binding of antibodies to receptor-bound viruses was detected by incubation with radioiodinated staphylococcal protein A (100,000 cpm, 30 min, 37°C). After washing, cells were detached from the plastic surface with 2% SDS and counted in a 1275 Minigamma gamma counter (Pharmacia Biotech). Results are the mean values of three experiments. All washes were done with DMEM plus 0.1% BSA. Protein A (Sigma) was labeled with $^{125}$I (Amersham Corp., UK) by the chloramine-T method (Greenwood *et al.,* 1963). Unbound $^{125}$I was removed by chromatography of the labeling mixture through a Sephadex PD-10 prepacked disposable column (Pharmacia Biotech). The specific activity of the $^{125}$I-labeled protein A was 5000 cpm/ng.

### Northern blot analysis

Confluent monolayers of astrocytes were washed with PBS and replenished with DMEM containing 0.25% FCS for 48 h in order to let the cells become quiescent. Quiescent cells were infected with the adenoviral vector Ad.$\beta$Gal as stated under Results. Total RNA was purified using the RNeasy minipurification kit (Qiagen Inc., Santa Clarita, CA). Ten micrograms per lane was electrophoresed on a formaldehyde-agarose gel and transferred to nylon membranes (Zeta-Probe, Bio-Rad Laboratories, Hercules, CA). A *v-fos* probe (Clontech, Palo Alto, CA) was labeled with [$^{32}$P]dCTP using the random-primed method (Boehringer Mannheim, Germany) and unincorporated radiolabel was removed with S-300 Micro-Spin columns (Pharmacia Biotech). The blots were exposed to Kodak X-OMAT film with an intensifying screen at −75°C. Hybridization to a human $\beta$-actin probe (Clontech) served as an internal control for loading.

### Viral neutralization by antibodies

Samples of Ad.$\beta$Gal at different concentrations in culture medium were incubated with increasing dilutions of the rabbit anti-adenovirus antiserum for 30 min at 37°C. Thereafter, the above incubation mixtures were used for infection of astrocyte cultures and further determination of *c-fos* induction.

### Apoptotic DNA fragmentation assay

Fetal calf serum starvation apoptosis was induced in 75-cm$^2$ flasks of confluent cultures by washing cells with

PBS and culturing in DMEM without FCS for 1 week. Other cultures were mock-infected as negative controls or infected at different m.o.i. with Ad.βGal. DNA was purified using the apoptotic DNA-ladder kit from Boehringer Mannheim and treated with DNase-free RNase (Boehringer Mannheim). Samples of 3 μg DNA per lane were separated by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining.

### Caspase-3 colorimetric determinations

Caspase-3 activity was determined in astrocyte cell lysates with the CaspACE assay system kit from Promega (Madison, WI). Briefly, infected cell cultures were washed with ice-cold PBS, lysed with cell lysis buffer, and centrifuged at 15,000*g* for 20 min after two cycles of freeze–thaw. Measurements of caspase-3 activity were performed according to the kit assay protocol in 96-well polystyrene plates (Nunc-Immuno plates, Nunc, Roskilde, Denmark) and the absorbance was read at 405 nm. To calculate the caspase-3-specific activity, a calibration curve using known amounts of the chromophore *p*-nitroaniline (pNA), released from the sustrate upon cleavage by the enzyme, was constructed. Specific activity was calculated as picomole of pNA liberated per hour per microgram of cell lysate extract using the formula provided with the CaspACE kit.

### Animal inoculations

Six-week-old Balb/c mice were anesthetized with Fluothane and injections made with a 10-μl Hamilton syringe, 1 mm right lateral and 2 mm rostral of the bregma. Five microliters of a suspension of Ad.βGal virus (titer = $1 \times 10^{10}$ PFU/ml) was infused at a rate of 1 μl every 5 s and the needle was maintained for an additional 10 s. Control injection mice received 5 μl of PBS. At suitable periods of time after injection, their brains were removed and samples were processed by immunochemistry.

### Immunocytochemical methods

Animals were perfused transcardially in all cases with 4% paraformaldehyde in PBS. After perfusion, the brains were removed, immersed in the same fixative for 3 h, and left overnight in PBS. Vibratome sections of 30–40 μm were processed free floating for immunohistochemistry. The sections (or the astrocyte cultures in 35-mm petri dishes) were incubated in the first primary antibody (mouse anti-β-galactosidase, 1:500, from Sigma) followed by biotinylated goat anti-mouse IgG (1:100, from Amersham) and Cy5-conjugated streptavidin (Amersham) diluted 1:1500. After several rinses in PBS, sections were stained for TUNEL. Astrocyte detection was accomplished with a monoclonal anti-glial fibrillary acidic protein (Sigma) diluted 1:500 followed by Cy5-conjugated goat anti-mouse IgG diluted also 1:500 (Amersham). The sections were examined in a Leica TCS NT confocal laser scanning microscope equipped with an argon/kryton-mixed gas laser with an excitation peak of 647 nm for Cy-5. Method specificity was controlled by omission of the primary antibodies.

### TUNEL assay

The detection of apoptotic cells was performed by using a TUNEL method. After several rinses, samples were processed for TUNEL using the *in situ* cell death detection kit following the manufacturer's instructions (Boehringer Mannheim). Stained cells were visualized by fluorescence microscopy in the confocal laser microscope at an excitation peak of 488 nm. Method specificity was controlled by omission of terminal deoxynucleotidyl transferase in the first step of the labeling.

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## REFERENCES

Allsopp, T. E., and Fazakerley, J. K. (2000). Altruistic cell suicide and the specialized case of the virus-infected nervous system. *Trends Neurosci.* **23,** 284–290.

Bergelson, J. M., Cunningham, J. A., Droguett, G., Kert-Jones, E. A., Krithivas, A., Hong, J. S., Horwitz, M. S., Crowell, R. L., and Finberg, R. W. (1997). Isolation of a common receptor for coxackie B viruses and adenoviruses 2 and 5. *Science* **275,** 1320–1323.

Berkner, K. L. (1988). Development for adenovirus vectors for the expression of heterologous genes. *Bio Techniques* **6,** 616–629.

Bossy-Wetzel, E., Bakiri, and Yaniv, M. (1997). Induction of apoptosis by the transcription factor c-jun. *EMBO J.* **16,** 1695–1709.

Byrnes, A. P., Rusby, J. E., Wood, M. J. A., and Charlton, H. M. (1995). Adenovirus gene transfer causes inflammation in the brain. *Neuroscience* **66,** 1015–1024.

Cartmell, T., Southgate, T., Rees, G. S., Castro, M. G., Lowenstein, P. R., and Luheshi, G. N. (1999). Interleukin-1 mediates a rapid inflammatory response after injection of adenoviral vectors into the brain. *J. Neurosci.* **19,** 1517–1523.

Chillon, M., Boch, A., Zabner, J., Law, L., Armentano, D., Welsh, M. J., and Davidsen, B. L. (1999). Group D adenovirus infect primary central nervous system cells more efficiently than those from group C. *J. Virol.* **73,** 2537–2540.

Choboczek, J., Ruigrok, R. W. H., and Cusack, S. (1995). Adenovirus fiber. *Curr. Top. Microbiol. Immunol.* **199,** 163–200.

Cua, D. J., Hutchins, B., LaFace, D. M., Stohlman, S. A., and Coffman, R. L. (2001). Central nervous system expression of IL-10 inhibits autoimmune encephalomyelitis. *J. Immunol.* **166,** 602–608.

Defer, C., Belin, M. T., Caillet-Boudin, M. L., and Boulanger, P. (1990). Human adenovirus-host cell interactions: A comparative study with members of subgroups B and C. *J. Virol.* **64,** 3661–3673.

Doerfler, W., and Bohm, B. (1995). The molecular repertoire of adenovirus. *Curr. Top. Microbiol. Immunol.* **199,** 83–93.

Dragunow, M., and Robertson, H. A. (1988). Brain injury induces c-fos protein(s) in nerve and glial-like cells in adult mammalian brain. *Brain Res.* **455,** 295–299.

Dragunow, M., Currie, R. W., Robertson, H. A., and Faull, R. (1989).

Heat-shock induces c-fos protein-like immunoreactivity in glial cells in adult rat brain. *Exp. Neurol.* **106,** 105–109.

González-Martín, C., De Diego, I., Fairen, A., Mellistrom, B., and Naranjo, J. R. (1991). Transient expression of c-fos during the development of the rat cerebral cortex. *Dev. Brain Res.* **59,** 109–112.

González-Martín, C., De Diego, I., Crespo, D., and Fairen, A. (1992). Transient c-fos expression accompanies naturally occurring cell death in the developing interhemispheric cortex of the rat. *Dev. Brain Res.* **68,** 83–95.

Green, M., Piña, M., and Kimes, R. C. (1967). Biochemical studies of adenovirus multiplication. XII. Plaque efficiencies of purified human adenovirus. *Virology* **31,** 562–566.

Greenwood, F. C., Hunter, W. M., and Glover, J. W. (1963). The preparation of ($^{131}$I)-labelled human growth hormone of high specific radioactivity. *Biochem. J.* **89,** 114–123.

Hagmeyer, B. M. H., König, I., Herr, R., Offinga, A., Zantema, A. J., van der Eb, P., Herrlich, P., and Angel, P. (1993). Adenovirus E1A negatively and positively modulates transcription of Ap-1 dependent genes by dimer-specific regulation of the DNA binding and transactivation activities of Jun. *EMBO J.* **12,** 3559–3572.

Hong, S. S., Karayan, L., Tournier, J., Curiel, D. T., and Boulanger, P. A. (1997). Adenovirus type 5 fiber knob binds to MHC class I $\alpha$2 domain at the surface of human epithelial and B lymphoblastoid cells. *EMBO J.* **16,** 2294–2306.

Hu, L., Hatano, M., Ruther, U., and Tokuhisa, T. (1996). Overexpression of c-Fos induces apoptosis of CD43+ pro-B cells. *J. Immunol.* **157,** 3804–3811.

Huang, S., Endo, R. I., and Nemerow, G. R. (1995). Upregulation of integrins $\alpha$v$\beta$3 and $\alpha$v$\beta$5 on human monocytes and T lymphocytes facilitates adenovirus-mediated gene delivery. *J. Virol.* **69,** 2257–2263.

Le Gal La Salle, G., Robert, J. J., Berrard, S., Ridoux, V., Stratford-Perricaudet, L. D., Perricaudet, M., and Mallet, J. (1993). An adenovirus vector for gene transfer into neurons and glia in the brain. *Science* **259,** 988–990.

Neve, R. L. (1993). Adenovirus vectors enters the brain. *Trends Neurosci.* **16,** 251–253.

Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., Munday, N. A., Raju, S. M., Smulson, M. E., Yamin, T.-T., Yu, V. L., and Miller, D. K. (1995). Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* **376,** 37–43.

Persson, R., Svensson, U., and Everitt, E. (1985). Virus-receptor interaction in the adenovirus system: Characterization of the positive cooperative binding of virions on HeLa cells. *J. Virol.* **54,** 92–97.

Previtali, S. C., Archelos, J. J., and Hartung, H.-P. (1997). Modulation of the expression of integrins on glial cells during experimental autoimmune encephalomyelitis. A central role for TNF-$\alpha$. *Am. J. Pathol.* **151,** 1425–1435.

Rauma, T., Tuukkanen, J., Bergelson, J. M., Denning, G., and Hautala, T. (1999). rab5 GTPase regulates adenovirus endocytosis. *J. Virol.* **73,** 9664–9668.

Rautenschlein, S., Suresh, M., and Sharma, J. M. (2000). Pathogenic avian adenovirus type II induces apoptosis in turkey spleen cells. *Arch. Virol.* **145,** 1671–1683.

Ridoux, V., Robert, J. J., Zhang, X., Perricaudet, M., Maller, J., and Le Gal La Salle, G. (1994). The use of adenovirus vectors for intracerebral grafting of transfected nervous cells. *Neuroreport* **5,** 801–804.

Rubio, N., and Cuesta, A. (1988). Receptors for Theiler's murine encephalomyelitis virus: Characterization by using rabbit antiviral antiserum. *J. Virol.* **62,** 4303–4306.

Rubio, N., De Felipe, C., and Torres, C. (1990). Theiler's murine encephalomyelitis virus-binding activity on neural and non-neural cell lines and tissues. *J. Gen. Virol.* **71,** 2867–2872.

Rubio, N., Rojo, P., and Torres, C. (1996). Overexpression of basal c-fos and c-jun but not of ras oncogenes after Theiler's murine encephalomyelitis virus infection of glial cells. *J. Neurovirol.* **2,** 167–174.

Rubio, N. (1997). Interferon-$\gamma$ induces the expression of immediate early genes c-fos and c-jun in astrocytes. *Immunology* **91,** 560–564.

Rubio, N., and Martin-Clemente, B. (1999). Theiler's murine encephalomyelitis virus infection induces early expression of c-fos in astrocytes. *Virology* **258,** 21–29.

Shih, D. S., Carrunth, L. M., Anderson, M., and Clements, J. E. (1992). Involvement of Fos and Jun in the activation of visna virus gene expression in macrophages through and AP-1 site in viral LTR. *Virology* **190,** 84–91.

Smeyne, R. J., Vendrell, M., Hayward, M., Baker, S. J., Miao, G. G., Schilling, K., Robertson, L. M., Curran, T., and Morgan, J. I. (1993). Continuous c-fos expression precedes programmed cell death *in vivo*. *Nature* **363,** 166–169.

Stevenson, S. C., Rollence, M., White, B., Weaver, L., and McClelland, A. (1995). Human adenovirus serotype 3 and 5 bind to two different cellular receptors via the fiber head domain. *J. Virol.* **69,** 2850–2857.

Stratford-Perricaudet, L. D., Makeh, I., Perricaudet, M., and Briand, P. (1992). Widespread long-term gene transfer to mouse skeletal muscles and heart via and adenovirus vector. *J. Clin. Invest.* **90,** 626–630.

Twu, J. S., Lai, M.-Y., Chen, D. S., and Robinson, W. S. (1993). Activation of protooncogene c-jun by the X protein of hepatitis B virus. *Virology* **192,** 346–350.

Verma, M., and Somia, N. (1997). Gene therapy—Promises, problems and prospects. *Nature* **389,** 239–242.

Weber, J. M., Talbot, B. G., and Delorme, L. (1989). The orientation of the adenovirus fiber and its anchor domain identified through molecular mimicry. *Virology* **168,** 180–182.

Wickham, T. J., Mathias, P., Cheresh, D. A., and Bemerow, G. R. (1993). Integrins $\alpha$V$\beta$3 and $\alpha$V$\beta$5 promote adenovirus internalization but not virus attachment. *Cell* **73,** 309–319.